ACTION OF THE PROTEINASES OF Clostridium oedematiens AND Clostridium perfringens ON HOMOLOGOUS AND HETEROLOGOUS TOXINS

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The production of highly effective preparations for the prophylaxis of gas gangrene is closely bound up with the solution of the problem of toxin formation, which includes three stages: 1) the synthesis of the toxin in the bacterial cell, 2) the mechanism of release of the toxin into the surrounding culture medium, and 3) the fate of the toxin in the culture medium. Practically no mention is made of the first two aspects of the problem in the literature. Attempts have been made to discover the possible causes of the lability of the toxins in the culture fluid. Several workers [1], for instance, have observed intensive proteolysis in concentrates of the toxin of Cl. perfringens, accompanied by a lowering of the titer of toxin when titrated by the lecithovitellin reaction.

The direct participation of the clostridial proteinases in the decomposition of homologous toxins can be established only by demonstrating their ability to interact after preliminary separation by means of suitable control experiments.

The object of the present investigation was to study the action of the proteinases of Cl. oedematiens and Cl. perfringens on homologous and heterologous toxins.

EXPERIMENTAL METHOD

The strains used in the investigation were Cl. oedematiens No. 794 (79), cultivated on media based on an acid hydrolyzate of casein, and Cl. perfringens No. BP6K28, cultivated on meat media.

The toxin of Cl. oedematiens was purified by precipitation with $CdCl_2$ to a content of 1% at pH 7.5, followed by elution with 4% K_2HPO_4 solution. The subsequent purification of the toxin was by gel filtration through Sefadex G-50 columns measuring 2.5 × 40 cm under a pressure of 5-8 cm water, at a flow velocity of 4-5 ml/180 sec. The fractions were collected by means of a type LKV automatic collector. The most active fractions of the toxin were adjusted with Tris buffer at pH 7.5 to give a 0.1 N solution. The toxin of Cl. perfringens was precipitated with ammonium sulfate to complete saturation; the resulting film was dissolved in a 0.01 N NaOH solution, passed through glass wool, and the filtrate applied to a Sefadex G-50 column. Fractionation of the Cl. perfringens toxin was carried out in the same way as that of Cl. oedematiens.

The protein content of the samples was determined by Lowry's method and their optic density was measured at 280 mμ in a type SF-4 spectrophotometer. The lethal dose of the toxins was determined by parallel titration experiments in vivo and in vitro in a chick embryo tissue culture, and expressed as cytotoxic units (CTU).

The proteinases were isolated as follows. A 24-h bacterial mass of Cl. oedematiens and Cl. perfringens was carefully rinsed with physiological saline and ground with powdered glass, and the resulting homogenate of bacterial cells was saturated with ammonium sulfate. The residue was separated by centrifugation and resuspended in physiological saline, to which an equal volume of chloroform was added, and the mixture agitated for 10-15 min and centrifuged for 25-30 min at 4000 rpm. The top aqueous layer was aspirated and the proteinase precipitated with an

TABLE 1. Action of Proteinase of Cl. oedematiens on Homologous Toxin

Composition of experimental sample	Initial sample		Incubation								
			2 h			4 h			6 h		
	toxicity (in CTU)	protein (in μg)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)
Toxin	2.500				2.000			2.000			2.000
Toxin + proteinase	2.500	1.490	120	0.8	2.000	280	18.8	2.000	560	37.6	50
Toxin + proteinase + cysteine 0.005 M	2.500	1.490	260	17.5	1.000	560	37.6	500	920	61.4	50
Toxin + trypsin	2.500	490	300	61.2	500	340	69.2	50	390	79.6	0

TABLE 2. Action of Proteinase of Cl. perfringens on Homologous Toxin

Composition of experimental sample	Initial sample		Incubation								
			2 h			4 h			6 h		
	toxicity (in CTU)	protein (in μg)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)
Toxin	200				200			200			200
Toxin proteinase + cysteine 0.005 M	200	5.600	1.000	18.2	100	1.840	32.9	50	2.360	42.1	20
Trypsin	200	2.800	550	19.6	100	720	25.7	0	920	32.8	0

TABLE 3. Action of Proteinases of Cl. oedematiens and Cl. perfringens on Heterologous Toxins

Composition of experimental sample	Initial sample		Incubation					
			3 h			5 h		
	toxicity (in CTU)	protein (in μg)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)	decrease of protein (in μg)	% of digestion	toxicity (in CTU)
Toxin of Cl. oedematiens + proteinase of Cl. perfringens + cysteine 0.005 M	4.820	200	7.0	15.3	100	1.860	36.5	20
Toxin of Cl. perfrigens + proteinase of Cl. oedematiens + cysteine 0.005 M	4.900	200	1.900	38.8	100	2.060	42.0	100

equal volume of acetone, cooled to −20°. The precipitate was centrifuged in the cold, dissolved in 0.1 N Tris buffer at pH 7.5, and again centrifuged at 6000 rpm for 20 min. The subsequent purification was by means of gel filtration through a Sefadex G-50 column.

The proteinase activity was determined by the splitting of casein at pH 7.5; the degree of breakdown was judged from the optic density at 280 mμ after precipitation of the unsplit proteins with trichloroacetic acid. The unit of

proteolytic activity was taken to be the proteinase activity splitting 0.1 mg protein in 1 ml of sample per hour at 37°. The proteinase activity in the original homogenate of Cl. oedematiens cells varied within wide limits – between 0.1 and 0.2 proteolytic unit. The activity of the final preparations was 70-90 units. The proteinase activity of Cl. perfringens was 0.21-0.25 proteolytic unit in the original homogenate of bacterial cells and 70-90 units in the final preparations.

The proteinases produced no lethal effect in the experiments in vivo and had no cytotoxic action in tissue culture in dilutions greater than 1:10.

EXPERIMENTAL RESULTS

Proteinase from Cl. oedematiens was incubated for 6 h with Cl. oedematiens toxin. The degree of proteolysis and the lethal properties of the samples were investigated after every 2 h of incubation. In parallel experiments cysteine was added to a concentration of 0.005 M to the proteinase of Cl. oedematiens as activator. In a series of experiments the relationship between Cl. oedematiens toxin and trypsin (bilyophilized, Hungary), of approximately the same strength as that of the proteinase of Cl. oedematiens when its activity was determined by the casein method, was investigated. The results of one such experiment are given in Table 1.

After the first 2 h of incubation, the proteinase of Cl. oedematiens, with the addition of cysteine, had split 17.5% of the proteins of the experimental sample. At the end of 6 h of incubation the degree of proteolysis reached 61.4%. At this time the lethal power of the toxin had fallen from 2500 CTU in the initial samples to 50 CTU in the final samples. The toxicity of the control samples not containing cysteine was reduced after 2 h of incubation from 2500 to 2000 CTU, and subsequently remained unchanged. In the samples not containing cysteine, the intensity of proteolysis at each period of determination was about half that in the samples containing cysteine. A fall in toxicity was observed only between the 4th and 6th hours of incubation.

It is clear from Table 1 that the toxin of Cl. oedematiens is sensitive to the action of trypsin.

The results of experiments to study the action of the proteinase of Cl. perfringens and trypsin on homologous toxin are given in Table 2. The experimental conditions were the same as in the experiments with proteinase of Cl. oedematiens.

The titer of the Cl. perfringens toxin remained practically unchanged during incubation for 6 h. Meanwhile the toxicity of the experimental samples containing proteinase of Cl. perfringens, activated with cysteine, fell from 200 CTU in the original samples to 20 CTU, and at this time 42.1% of the protein was digested. In the experimental samples containing trypsin instead of proteinase, after incubation for only 4 h the percentage of toxicity had fallen to zero and 25.7% of the protein was digested. Evidently trypsin, like the homologous proteinases, possesses the ability to split the lethal factors of Cl. oedematiens and Cl. perfringens. It was of interest to examine the action of these two proteinases on the heterologous toxins. The results of one such experiment are given in Table 3.

Proteinase of Cl. perfringens, in the presence of 0.005 M cysteine, split 36.5% of the proteins of the experimental sample during incubation for 5 h. The toxocity of the experimental sample had fallen by 90% at the end of incubation for 5 h. As a rule the decrease in toxicity of the samples in the experiments to study the action of proteinase of Cl. oedematiens on Cl. perfringens toxin was smaller. After incubation for 5 h the toxicity of was lowered by only 50% (see Table 3), although the degree of protein digestion was slightly greater than in the case of the action of the proteinase of Cl. perfringens on Cl. oedematiens toxin. It should be noted that the Cl. perfringens toxin used in our experiments contained proteinase, but its activity was extremely low and could not have influenced the experimental results significantly.

Hence the toxins of Cl. oedematiens and Cl. perfringens apparently are capable of being split by both homologous and heterologous proteinases and, in particular, by trypsin. A similar phenomenon has been described during the study of the action of the proteinase of group A streptococci on the M antigen [2-4].

SUMMARY

The author elaborated a purifying method for endoproteinases of Cl. oedematiens and Cl. perfringens by precipitation with ammonium sulfate with a subsequent treatment with chloroform and acetone and gel filtration through sefadex G-50. The action of proteinases on partially purified homologous and heterologous toxins was accompanied by a marked drop of toxicity (titration in vivo and in vitro – on tissue culture of chick embryo). Trypsin action on the toxins studied was analogous to the effect produced by Cl. oedematiens and Cl. perfringens endoproteinases with a simultaneous splitting of a considerable proportion of proteins.

LITERATURE CITED

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All abbreviations of periodicals in the above bibliography are letter-by-letter transliterations of the abbreviations as given in the original Russian journal. *Some or all of this periodical literature may well be available in English translation.* A complete list of the cover-to-cover English translations appears at the back of this issue.